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Original Article

Root Protein Interactomics of Salt Stress-Induced Proteins of Wheat Genotypes KH-65 (Salt-Tolerant) and PBW-373 (Salt-Susceptible)

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Abstract

Introduction: Wheat crop is moderately tolerant to salt stress and is considered as an excellent system to study salt stress tolerance despite its genetic complexity. In the present study, the top ten biological processes in a root proteome were mapped for Protein-Protein Interaction (PPI) networks and analyzed to examine the effect of salt stress on wheat cultivars KH-65 and PBW-373.

Materials and Methods: NaCl salinity treatment 0 and 300 mM NaCl was performed on a three-leaf stage plant. Roots proteins were analyzed by liquid chromatography-mass spectroscopy. Proteins were grouped according to GENE ontology terms for biological process and arranged in descending order. Interactome analysis was done through the STRING database.

Results: Interacting root proteins of tolerant line KH-65 show a comparatively higher number of nodes, edges, and interacting proteins than the sensitive line, PBW-373. The number of proteins whose expression was positively induced upon salinity stress was significantly higher in the roots of salinity-tolerant KH-65 than that of the PBW-373 roots. Moreover, the fold induction too was also high in the tolerant line. Similarly, the number of participant proteins in an interaction network of the KH-65 roots was higher than that of the PBW-373 cultivar.

Conclusions: This analysis provides valuable information in elucidating the molecular mechanism associated with salt stress response in wheat seedlings' roots. The observation is correlated with the efficient salt tolerance capacity of KH-65. The higher expressing proteins in interaction networks may be seen under the increased salt tolerance capabilities of salt-tolerant KH-65 line.

Keywords: *Triticum Aestivum*, KH-65, PBW-373, Salt Stress, Protein-Protein Interaction

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Introduction

Plants possess complicated regulatory mechanisms that may respond to varying conditions of the environment and overcome abiotic stresses, including salinity.[1] Wheat is an important cereal in the world nutrition scenario and is commonly consumed in bread as a good source of carbohydrates, proteins, vitamins, minerals, valuable phytochemicals, and other dietary components.[2,3] It is among the most cultivated cereal crops globally[4] grown on 22% arable land. The cultivation area of wheat covers broad geographical conditions, including arid and semi-arid regions where production is primarily limited by salinity stress. Salinity affects the overall development of plants, including growth, development, and yield. Plants survive in different types of environmental conditions through a wide range of genetic variations. Plants change gene expression patterns and protein accumulation under stress conditions.[5,6] The impact of salt stress on the metabolism, altered gene expression, and protein profiling has been reported.[7-9] These changes in plants' expression patterns allow them to withstand stress conditions, which leads to stress tolerance. Expression profiling can define both sensitive and tolerant genotypes. Therefore, the study of expression profiling is essential for investigating plant response towards stress,[7] as differential expressions among cultivars were known to provide a different level of tolerance.[10] To expand the studies of plant stress responses and adaptation mechanisms, analysis of stress-induced proteins is critical in proteomic studies as it provides insight into the intricate mechanism of stress mitigation. At the cellular level, the proteins interact to function as "molecular machines" and establish dynamic physicochemical connections to regulate biological functions. PPI are essential to understand the complex molecular relationships during stress[11]. Alterations in the protein profiles and their interactome studies in rice during abiotic stresses have been investigated.[12] However, very few studies have been conducted on PPI networks of wheat seedlings under salt stress. These interaction networks may

have considerable significance in understanding plant systems' stress responses.[13] The interactome- guided prediction can identify novel regulators of stress tolerance.[14] The combination of focused interactome and system analyses can significantly progress towards elucidating agronomic importance traits' molecular basis.

Wheat has several cultivars with diverse salinity tolerance. Two contrasting wheat genotypes are used in the present investigation, of which KH-65 is salinity tolerant while PBW-373 is sensitive. The present study is designed to perform in silico analysis of the combinatorial root interactome of differentially expressed proteins of KH-65 (salt-tolerant) and PBW-373 (salt-susceptible).

Materials and Methods

Plant Material, Growth Conditions, and Experimental Details

The seeds of contrasting wheat genotypes, Kharchia-65 (Salt-tolerant) and PBW-373 (Salt-susceptible), were obtained from the Indian Institute of Wheat and Barley Research, Karnal, India. Wheat seeds were sterilized with 0.1% $HgCl_2$ for 2 min, then rinsed with double distilled water and germinated on autoclaved sand. Upon germination, the seedlings were then transferred to a hydroponic culture medium for 48 h inside a growth chamber. The half-strength modified Hoagland's solution[15] was used for growing the plants at 20 °C, for photoperiods of 16:8 hour day-night cycle, and intensity of light 2000 lux.[15,16] The salinity treatments to the seedlings were given at the three-leaf stage. Against the control (containing half-strength modified Hoagland solution alone), 300 mM of saline concentration (NaCl) was applied for 48 h. Proteins extraction was carried out using the phenol extraction method reported by Faurobert et al.[17] Bradford reagent was used for the estimation of total soluble proteins.[18] Each sample's absorption was recorded at 595 nm on a spectrophotometer (SYSTRONICS, Smart UV VIS Double Beam Spectrometer with Graphic LCD-Type 2203). The samples were analyzed using a nano-flow liquid chromatography (EASY-nLC 1000 system, Thermo-Fisher Scientific) pre-optimized for proteins and peptides separation. Mass spectral data were recorded by the selection of abundant precursor ions in the survey scan. The enzyme used for the generation of peptides was trypsin/P with a maximum of two missed cleavages. False discovery rate and spectrum match for proteins were set at 0.01 FDR.

Data Analysis

Differential analysis between PBW-373 and KH-65 lines was done by deploying the protein abundance values in each sample [PBW-373 (Control), PBW-373 (Treated), KH-65 (Control), and KH-65 (Treated)]. The abundance value of proteins was filtered, and missing values were then imputed using a normal distribution. The abundance values were Log2 transformed, succeeded by Z-score standardization for normalization of data. Statistical significance was conferred through ANOVA, and FDR<0.05 was considered for statistically significant proteins. The differential analysis was also performed separately between treatment and control for both the lines, KH-65 and PBW-373. Student T-test was applied to the data. Statistical significance was tested against the FDR<0.05; in-house R scripts were used for visualization. Gene Ontology terms in the identified proteins were annotated from the Uniprot database using Accession IDs for the functional annotation of categories. Proteins were grouped according to GENE ontology terms for biological process and arranged in descending order.

Interactome Analysis Through STRING Database

The STRING protein-protein interaction networks functional enrichment analysis was used to drive the confidence analysis of PPI Networks for all differentially expressed proteins (DAPs). The top ten biological processes viz. translation, Intracellular protein transport, vesicle-mediated transport, transcription, biosynthetic process, protein folding, carbohydrate metabolism, glycolysis, lipid transport, and hydrogen peroxide catabolic process were analyzed in KH-65 and PBW-373 roots after a 48 h exposure of salinity stress.[19] String 11.0 version was used for analysis (https://string-db.org/). The PPI with confidence scores higher than 0.7 were shown. To enhance the validity of different groups of interactions which are significantly enriched in the pathways, only experimentally proven active interaction sources were considered for the present investigation.

Results and Discussion

The PPIs in the roots of wheat lines, KH-65 and PBW-373 under salt stress, are presented in Figures 1 and 2. Analysis of KH-65 root interactome gives us 108 nodes, 425 edges, and five clusters of interacting proteins consisting of 66 proteins. The interactome of the sensitive line, PBW-373, had a comparatively lesser number of nodes, edges and interacting clusters of proteins than the tolerant line, KH-65. The root interactome of PBW-373 consists of only 75 nodes, 344 edges, and 43 interacting proteins in five interactions. In a biological system, a cascade of reactions co-occurs to manifest a biochemical pathway. Each step of a cascade involves enzymes. Thus proteins must act as a molecular machine.[20] The details of the wheat proteins of the top ten biological processes that participated in the interactome network are presented in Supplementary Tables S1 and S2. Details of top upregulated and down regulated proteins of both the lines are given in Tables 1-4. This combinatorial interactome depicts significant changes in the PPI networks in the root of salt stress-induced differentially expressed proteins.

Figure 1. Combinatorial Root Interactome of Differentially Expressed Proteins (DEP's) in Tolerant Line KH-65.

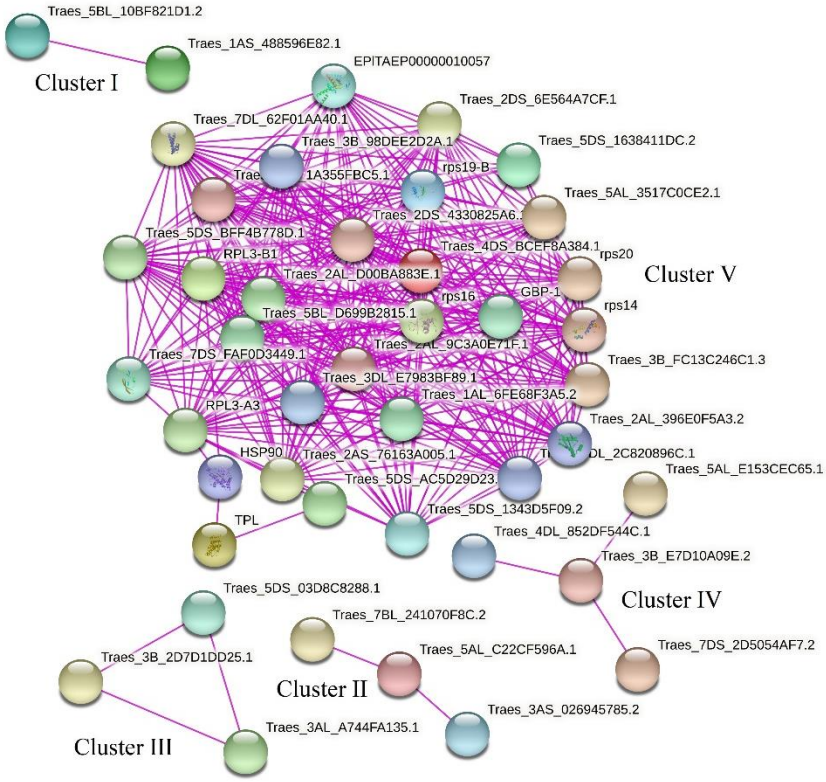

Figure 2. Combinatorial Root Interactome of Differentially Expressed Proteins (DEP's) in Sensitive Line PBW-373.

Table 1. Top 15 Upregulated Proteins and Number If Their Interacting Partners in Root Proteins of KH-65 Interactome

S. No.	KH-65, Root Protein	Coding Name	Biological Process	Common or Unique Proteins	Fold Change	Interacting Partners
1	Vacuolar protein sorting-associated protein 41 homolog	Traes_2AL_0B9E1F386.1	Vesicle mediated transport	Unique	6.64	4
2	VHS domain-containing protein	Traes_3AS_026945785.2	Intracellular protein transport	Common protein	6.64	1
3	Clathrin heavy chain	Traes_5AL_C22CF596A.1	Vesicle mediated transport	Unique	6.64	4
4	Auxin-responsive protein	Traes_5BL_EC006AD0C.1	Transcription	Unique	6.64	1
5	WD_REPEATS_REGION domain-containing protein	Traes_6AS_1A54BB177.1	Transcription	Unique	6.64	3
6	CNH domain-containing protein	Traes_4AL_96E6567CA.1	Intra cellular protein transport	Unique	3.45	3
7	TOPLESS	TPL	Transcription	Common protein	1.73	3
8	Ribosomal protein L15	Traes_2AS_76163A005.1	Translation	Common protein	1.65	24
9	MHD domain-containing protein	Traes_6BL_470ECDCDF.1	Vesicle mediated transport	Unique protein	1.44	3
10	Coatomer subunit alpha	Traes_5AL_E153CEC65.1	Intracellular protein transport	Common protein	1.26	2
11	60S ribosomal protein L18a	Traes_1AL_6FE68F3A5.2	Translation	Common protein	0.86	25
12	S4 RNA-binding domain-containing protein	Traes_2DS_6E564A7CF.1	Translation	Common protein	0.85	25
13	AP-4 complex subunit epsilon	Traes_3B_1DB17F0DF.1	Vesicle mediated transport	Unique	0.82	2
14	VAMP like putative proteins belongs to the synaptobrevin family	Traes_3B_2D7D1DD25.1	Vesicle mediated transport	Common protein	0.73	7
15	Ribosomal protein L3-A3	RPL3-A3	Translation	Common protein	0.61	28

The combinatorial root interactome of KH-65 is a complex interactome with the participation of 66 proteins forming PPIs. The cluster-I, the smallest cluster involved in the interaction of two proteins, namely GrpE protein homolog and J domain-containing protein, was involved in protein folding. The link of these proteins with enhancing tolerance and managing stress via a function in translocation, proper folding, and removing stress-damaged proteins has been elucidated in many studies.[21,22] The cluster-II involved an interaction network of three proteins. Two proteins, namely, Rab18s and Guanosine nucleotide diphosphate dissociation inhibitor, were involved in intracellular protein transport, and one protein called Small GTP-binding protein was involved in the vesicle-mediated transport. All the three proteins in this cluster are found in the down regulated state.

Similarly, Cluster-III and Cluster-IV consisted of ten proteins. Each cluster consisted of intracellular protein transport or vesicle-mediated transport. Most of the proteins in these two clusters were found upregulated in response to salt stress. Among these, the upregulated proteins of cluster-III were CNH domain-containing protein (involved in vacuolar protein sorting),[23] GTPase SAR 1 (involved in protein sorting and secretory trafficking),[24] and Putative SNAP receptor protein (involved in the maintenance of cellular homeostasis, membrane fusion, and transport vesicles and salinity resistance)[25] vacuolar protein sorting-associated protein 41 homologs (involved in the regulation of transport and provides salinity tolerance),[26] t-SNARE coiled-coil homology domain-containing protein (involved in vesicle fusion, docking and intracellular protein transport [W5FLZ1]), and VAMP like putative protein (involved in protein transport through vesicles).[27]

Whereas, the list of the upregulated proteins of cluster IV included coatomer subunit α, β and γ proteins (involved in retrograde protein transport),[28] Coatomer subunit gamma, and VHS domain-containing protein (involved in protein sorting and secretory trafficking),[24] AP-4 complex subunit epsilon (involved in cell vesicle transport),[29] Clathrin heavy chain (mediates endocytosis),[30] and MHD domain-containing protein (increased secretion via helping in vesicle priming).[31] The transport proteins have been reported to be salinity stress-responsive in the tolerant genotype.[22,32]

The largest cluster, Cluster-Vof interacting proteins that belong to biological processes, namely, intracellular protein transport, hydrogen peroxide catabolic process, transcription, and translation, is the dominant one. In this cluster, proteins involved in glycolysis, transcription, and translation were upregulated. Li et al.[8] also reported a more extensive PPI network as an indicator of better resistance capacity of tolerant line.[8]

The upregulated proteins of Cluster-V included Pyruvate kinase that is involved in the glycolysis pathway. The auxin-responsive protein, WD_REPEATS_REGION domain-containing protein, and TOPLESS which involved in the regulation of transcription. The HATPase_c domain-containing protein is involved in protein folding. The Ribosomal_L2_C domain-containing protein, S4 RNA-binding domain-containing protein, Ribosomal proteins L3-A3, L3, L15, 60S ribosomal protein L18a, and TFIIB-type domain-containing protein are involved in translation.

The combinatorial root interactome of PBW-373 consists of five clusters. The smallest one, Cluster-I, had an interaction network of only two down-regulated proteins, GrpE protein

Table 2. Top 15 Downregulated Proteins and Number If Their Interacting Partners in Root Proteins of KH-65 Interactome

S. No.	KH-65	Coding Name	Biological Process	Common/Unique	Fold Change	Interacting Partners
1	RPOLD domain-containing protein	Traes_4BL_1638411DC.2	Translation	Unique protein	-4.99	10
2	30S ribosomal protein S14, chloroplastic	rps14	Translation	Common protein	-4.3	28
3	Ribosomal protein L19	Traes_2DS_2697C9D0A.1	Translation	Common protein	-3.7	23
4	30S ribosomal protein S2, chloroplastic	EPlTAEP00000010057	Translation	Common protein	-3.65	27
5	Thioredoxin M-type, chloroplastic	Traes_5BS_B72CD04F2.1	Hydrogen peroxide catabolic process	Unique protein	-3.56	1
6	Protein transport protein Sec61 subunit beta	Traes_4DS_BCEF8A384.1	Intracellular protein transport process	Common protein	-3.27	23
7	30S ribosomal protein S19, chloroplastic	Traes_3DL_262C70465.1	Translation	Common protein	-3.17	23
8	2-Cys peroxiredoxin BAS1, chloroplastic	Traes_2BL_E6F86DAFA.1	Translation	Unique protein	-2.91	4
9	50S ribosomal protein L33, chloroplastic	Traes_5DS_FAF0D3449.1	Translation	Common protein	-2.9	15
10	Protein VACUOLELESS1	Traes_3AL_A744FA135.1	Intra cellular protein transport	Unique protein	-2.86	4
11	Catalase	CAT1	Hydrogen peroxide catabolic process	Common protein	-2.74	1
12	Not3 domain-containing protein	Traes_1AL_C550E0E88.1	Transcription	Unique protein	-2.24	25
13	Protein disulfide-isomerase	PDI2	Protein folding	Common protein	-1.7	1
14	Ribosomal_L28e domain-containing protein	Traes_3DL_E7983BF89.1	Translation	Common protein	-1.68	23
15	Ribosomal_S10 domain-containing protein	Traes_5DS_BFF4B778D.1	Translation	Common protein	-1.61	26

homolog (involved in managing stress via a function in translocation)[21] and J domain-containing protein (involved in proper folding)[33] that are involved in protein folding. Similarly, two more clusters, Cluster II and III, were also present in the three proteins' interaction networks. Of these, one cluster had an upregulated clathrin heavy chain protein (mediates endocytosis),[30] and two down-regulated proteins, clathrin light chain and VHS domain-containing protein that play a role in intracellular protein transport.

Another three protein interaction clusters consist of one protein belonging to vesicle-mediated transport (VAMP-like protein) and two belonging to intracellular protein transport (VACUOLELESS1; t-SNARE coiled-coil homology domain-containing protein); all the three proteins were repressed down regulated due to salinity stress. Cluster IV had an interaction network of four proteins, of which one central protein, Coatomer subunit alpha (involved in protein sorting and secretory trafficking)[24] interacted with the other three proteins, AP-1 complex subunit gamma (mediates vacuolar targeting),[34] Coatomer subunit alpha[28] and Coatomer subunit beta (involved in protein transport from ER to the cis-trans compartment).[35]

The cluster-V was the cluster of the most extensive interaction network comprising 31 proteins belonging to the biosynthetic process, intracellular protein transport, transcription, glycolysis, protein folding, and translation. Among these,

Table 3. Top 12 Upregulated Proteins and Number If Their Interacting Partners in Root Proteins of PBW-373 Interactome

S. No.	KH-65 Root Proteins	Coding Name	Biological Process	Common or Unique Proteins	Fold Change	Interacting Partners
1	AP-1 complex subunit gamma	Traes_7DS_2D5054AF7.2	Intra cellular protein transport	Unique protein	6.64	1
2	30S ribosomal protein S19, chloroplastic	Traes_3DL_262C70465.1	Translation	Common protein	6.64	23
3	RPOLD domain-containing protein	Traes_4BL_1638411DC.2	Translation	Common protein	5.7	9
4	Ribosomal protein L3	RPL3-B1	Translation	Common protein	1.49	27
5	Coatomer subunit beta	Traes_3B_E7D10A09E.2	Intra cellular protein transport	Common protein	1.18	3
6	Coatomer subunit alpha	Traes_5AL_E153CEC65.1	Intra cellular protein transport	Common protein	1.16	1
7	Ribosomal protein L15	Traes_2AS_76163A005.1	Translation	Common protein	1.05	22
8	TOPLESS	TPL	transcription	Common protein	0.91	2
9	Coatomer subunit alpha	Traes_4DL_852DF544C.1	Vesicle mediated transport	Common protein	0.78	1
10	Clathrin heavy chain	Traes_5AL_C22CF596A.1	Intracellular protein transport	Common protein	0.76	2
11	Ribosomal protein L3-A3	RPL3-A3	Translation	Common protein	0.74	26
12	SYP71 protein	Traes_5DS_BFF4B778D.1	Translation	Unique protein	0.68	26

Table 4. Top 15 Downregulated Proteins and Number If Their Interacting Partners in Root Proteins of PBW-373 Interactome

1	Ribosomal protein L19	Traes_2DS_2697C9D0A.1	Translation	Common protein	-4.34	23
2	30S ribosomal protein S14, chloroplastic	rps14	Translation	Common protein	-4.3	27
3	RRF domain-containing protein	Traes_2AL_D00BA883E.1	Translation	Unique protein	-4.29	18
4	HATPase_c domain-containing protein	Traes_5DS_AC5D29D23.1	Protein folding	Common protein	-3.98	3
5	VAMP-like protein	Traes_3B_2D7D1DD25.1	Vesicle mediated transport	Common protein	-3.09	2
6	50S ribosomal protein L33, chloroplastic	Traes_5DS_FAF0D3449.1	Translation	Common protein	-2.9	17
7	Ribosomal_L28e domain-containing protein	Traes_3DL_E7983BF89.1	Translation	Common protein	-2.6	22
8	30S ribosomal protein S2, chloroplastic	EPITAEP00000010057	Translation	Common protein	-2.58	25
9	Clathrin light chain	Traes_7BL_241070F8C.2	Intracellular protein transport	Common protein	-2.53	1
10	GrpE protein homolog	Traes_5BL_10BF821D1.2	Protein folding	Common protein	-2.42	1
11	Putative ribosomal protein S18	Traes_5BL_1A355FBC5.1	Translation	Common protein	-2.15	27
12	Ribosomal_S13_N domain-containing protein	Traes_7DL_62F01AA40.1	Translation	Common protein	-2.07	25
13	J domain-containing protein	Traes_1AS_488596E82.1	Protein folding	Common protein	-1.92	1
14	Protein transport protein Sec61 subunit beta	Traes_4DS_BCEF8A384.1	Intracellular protein transport	Common protein	-1.91	22
15	KOW domain-containing protein	Traes_3B_FC13C246C1.3	Translation	Common protein	-1.9	26

Among these, TOPLESS, 30S ribosomal protein S19, chloroplastic, SYP71 protein, Ribosomal protein L3-A3, RPOLD domain-containing protein, Ribosomal protein L3 protein, and phosphotransferase belongs to transcription, translation, and glycolysis pathways. The majority of proteins belong to transcription, intracellular protein transport, protein folding, and translation pathways.

The number of proteins whose expression was positively induced upon salinity stress was significantly higher in the roots of salinity-tolerant KH-65 than that of the PBW-373 roots. Only 12 proteins could be recorded as upregulated among the top 10 biological processes of the roots of PBW-373 (Tables 1-4). Moreover, the fold induction too was also high in the tolerant line. Similarly, the number of participant proteins in an interaction network of the KH-65 roots was higher than that of the PBW-373 cultivar. The observation may be correlated with the efficient salt tolerance capacity of KH-65. A higher number of high expressing proteins in interaction networks has also been associated with the salt tolerance capability.[14]

The upregulated proteins of the tolerant cultivar included pyruvate kinase. Glycolytic proteins have been proposed to maintain energy balance in salt-tolerant plants during stress conditions.[36] Similarly, the tolerant line was found to respond to salt stress by modulating various transcription regulatory proteins. The finding was in conformation with other reports.[37] A transcription regulatory protein, Auxin responsive protein, has also been associated with tolerance mechanism via suppression of auxin-regulated genes.[38] Other proteins in the interactomes whose role in coping with salinity stress has been reported including, Heat shock protein 90,[39] HATPase c domain-containing protein,[40] S4 RNA-binding domain-containing protein,[41] 60S ribosomal protein L18a,[42] TFIIB-type, and WD repeats region domain-containing proteins.[38]

Root directly interacts with the soil and is the first site to counter the soil's high salt concentration.[43] The robust response of the roots of the resistant line was expected. All stress conditions induce a cascade of reactions involved in various physiological processes to counter-balance the damage due to the stress condition. The plants are known to counter salinity stress by causing various biological phenomena that include altered gene expression to modulate growth and development, ion transportation and storage of excess ions, and production of compatible solutes like anti-stress proteins, including chaperons and HSPs.[44] All these processes involve the activation of a great deal of plant machinery that may reflect the upregulation of numerous proteins that act in tandem to manifest the tolerance mechanism.

Conclusion

The study provides an insight into a relation between protein-protein interaction networks for a group of interacting proteins amid salt stress. A higher number of high expressing proteins in interaction networks in the salt-tolerant variety than the sensitive one may be seen under the light of more increased salt tolerance capabilities of the Salt-tolerant KH-65 line. Further in-depth studies are needed to validate the findings.

Authors' Contributions

RY, ARS, and NPS contributed equally to this study.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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Supplementary Materials

Supplementary material includes Tables S1 to S2.

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